Marrow Accessory Cell Infection and Alterations in Hematopoiesis Accompany Severe Neutropenia During Experimental Acute Infection With Feline Immunodeficiency Virus

By Michael L. Linenberger, Amy M. Beebe, Niels C. Pedersen, Janis L. Abkowitz, and Satya Dandekar

Severe neutropenia and bone marrow (BM) morphologic abnormalities occur during experimentally induced primary infection with feline immunodeficiency virus (FIV), a lentivirus biologically similar to human immunodeficiency virus (HIV).

To further characterize the mechanisms involved in this acute infection model of lentivirus-induced BM suppression, peripheral blood counts, histologic BM studies, and BM culture assays were performed on 12 cats that underwent necropsy at regular intervals postinoculation (PI) with FIV Petaluma. Plasma viremia developed at week 3 PI and neutropenia was initially detected at week 6 PI. Low neutrophil counts, but normal hematocrits and platelet counts, persisted through week 12 PI. Infected BM mononuclear cells and megakaryocytes were identified by in situ hybridization assays for FIV nucleic acids in BM sections of cats that underwent necropsy at weeks 4 to 12 PI, correlating with detection of soluble FIV p24 antigen and identification of infected mononuclear and macrophage cells in BM buffy-coat cell cultures from these cats. At weeks 1.5 to 4 PI, the mean frequencies (number per 10^6 BM mononuclear cells) of erythroid progenitors (erythroid colony-forming units [CFU-E] and erythroid burst-forming units [BFU-E]) and granulocyte/macrophage progenitors (CFU-granulocyte/macrophage [CFU-GM]) were increased to 508 ± 74, 143 ± 24, and 110 ± 17, respectively (n = 5 cats) as compared with controls (172 ± 24, 86 ± 26, and 44 ± 10; n = 3 cats; P < .02), and the percentages of progenitors in the DNA-synthetic phase of the cell cycle were equivalent to controls. In contrast, the progenitor frequencies at weeks 6 to 12 PI were significantly decreased (72 ± 16, 43 ± 6, and 19 ± 4, respectively; n = 7 cats; P < .01), and these progenitors were more frequently in S-phase. Autologous serum significantly inhibited (P < .05) the growth of CFU-GM in 6 of 9 cats and failed to support the maximal growth of BFU-E in 4 of 9 cats studied at weeks 4 to 12 PI, whereas no such abnormalities were observed in colony assays containing autologous sera from control cats (n = 3) or cats studied at weeks 1.5 or 3 PI (n = 3). In comparison, sera from FIV-infected cats did not inhibit the growth of normal, allogeneic progenitors. However, FIV serum frequently failed to support maximal in vitro growth of normal CFU-GM as compared with uninfected allogeneic sera, further suggesting a lack of progenitor growth-promoting substances in infected cat sera. Together, these studies show that neutropenia induced by experimental acute FIV infection is associated with plasma viremia, with the appearance of viral-infected BM accessory cells, and with alterations in progenitor frequencies, cell cycle kinetics, and in vitro growth in autologous sera. Because these virologic, hematologic, and hematopoietic abnormalities resemble those associated with late-stage HIV infection, acute FIV infection may provide an excellent model to characterize the lentiviral and host cell factors relevant to mechanisms of BM suppression in the acquired immunodeficiency syndrome.

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PRIMARY AND LATE STAGES of infection with the human immunodeficiency virus (HIV) are associated with peripheral blood cytopenias. However, the causal role of HIV in these hematologic disorders remains unclear. Neutropenia and thrombocytopenia are noted during symptomatic acute HIV infection, whereas decreased peripheral blood counts and bone marrow (BM) morphologic abnormalities frequently develop in patients with the acquired immunodeficiency syndrome (AIDS).

Because high levels of plasma viremia and antigenemia occur during both of these clinical stages of infection, the pathogenesis of HIV-associated BM suppression could be directly related to a high rate of viral replication. By extension, studies of hematopoiesis during primary infection might identify viral and/or host cell factors relevant to the hematologic disorders associated with AIDS. Such studies during acute infection would avoid the potential confounding effects on the BM of opportunistic infections, profound immunosuppression, or neoplasia. No systematic studies have been performed on the BM of patients with primary (acute) HIV infection most likely because of the sporadic occurrences and unpredictable identification of patients with this presumptive diagnosis.

Feline immunodeficiency virus (FIV) infection in cats provides an excellent animal model to study lentiviral-induced hematologic disorders. FIV causes a long-latency immunodeficiency syndrome (feline AIDS), which is frequently associated with anemia, neutropenia, and BM morphologic abnormalities that resemble those in patients with AIDS.

Although BM culture studies on hematologically normal cats with chronic, asymptomatic FIV infection show normal hematopoietic progenitor frequencies, cell cycle kinetics, and in vitro growth characteristics, studies of BM from one FIV-seropositive cat with feline AIDS and chronic neutropenia suggested that a serum factor inhibited myeloid progenitor growth. Together, these data suggest that FIV-induced BM suppression, similar to HIV, is probably caused by factors associated with increased viral replicative activity and/or increased immunosuppression. Observations that experimental acute infection with FIV induces severe neutropenia, and in some cases BM myeloid dysplasia, further support the hypothesis that high levels of viral expression alone may be causally related to FIV-associated hematologic abnormalities.

To further characterize the host cell, viral, and humoral factors relevant to the pathogenesis of BM suppression associated with feline AIDS, a project was designed to study the BM suppression associated with experimental acute FIV infection in cats, in hopes of providing a model to elucidate the mechanisms of BM suppression.

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factors involved in lentivirus-induced BM suppression, histologic in situ hybridization assays and cell culture studies were performed on the BM from 12 cats that underwent necropsy at 1- to 2-week intervals after inoculation with FIV Petaluma. These studies were designed to determine whether the development of neoplasia correlated with productive or latent BM cell infection with FIV and with alterations in hematopoietic progenitor frequencies and/or in vitro growth characteristics.

MATERIALS AND METHODS

Study animals. FIV infection, and in vivo assays. Specific pathogen-free (SPF) domestic cats, aged 16 to 20 weeks, were obtained from the breeding colony of the Feline Retrovirus Research Laboratory (University of California, Davis, CA) and were inoculated intraperitoneally with 1.0 mL of whole heparinized blood from an SPF chronic carrier of the Petaluma strain of FIV (cat no. 5000). Donor cat no. 5000 was asymptomatic but had persistent CD4+ T lymphocytes. Inoculated cats were housed in facilities of the Animal Resource Services at the University of California, Davis.

Plasma viremia was determined by the ability of 100 µL plasma to productively infect 10^6 normal cat peripheral blood (PB) mononuclear cells (MNCs) stimulated with concanavalin A. Culture supernatants were tested for the presence of FIV p24 by antigen-capture enzyme immunoassay (ELISA). Antibodies against FIV p24 core antigen were detected using an ELISA. Complete blood counts, including hemocrit, platelet count, and total and differential white blood cell counts, were determined biweekly and on the day of necropsy. Two animals were euthanized at 1- to 2-week intervals postinoculation (PI) by intravenous barbiturate overdose. PB and heparinized BM samples were taken at necropsy for cell studies. BM tissue was also fixed in 10% buffered formalin and embedded in paraffin for later in situ hybridization and morphologic assays. Complete in vitro studies were performed on 2 cats each at weeks 1.5 (nos. 5108, 5123), 4 (nos. 5106, 5127), 6 (nos. 5105, 5125), 10 (nos. 5122, 5128), and 12 (nos. 5096, 5097) PI, whereas only 1 cat was studied at weeks 3 (no. 5107) and 8 (no. 5129) PI.

Uninfected control animals included 1 age-matched SPF domestic cat that underwent necropsy at mid-study (no. 5104) and 2 age-matched non-SPF domestic cats from which PB and BM samples were taken after anesthesia with ketamine.

Detection of FIV RNA and proviral DNA in BM cells by in situ hybridization. Formalin-fixed, paraffin-embedded BM biopsy specimens were tested by RNA in situ hybridization for the presence of RNA alone and by RNA/DNA in situ hybridization for the presence of DNA and/or RNA. Progenitor-derived colonies were established containing BMMNCs from infected cats with either 2XSSC at 42°C, dehydrated, and dipped in NTB2 emulsion (Eastman Kodak Co, Rochester, NY). After exposure for 7 to 12 days at 4°C, slides were developed and counterstained with Mayer's hematoxylin (Sigma). Adherent stromal cells and nonadherent cells were fixed for 1 hour in 10% buffered formalin before in situ hybridization and blocked for 2 hours at 42°C with hybridization solution without probe before hybridization with the radioactively-labeled FIV DNA probe. Each in situ hybridization experiment included uninfected control tissues, stromal cell cultures, or nonadherent cells. A scattered background of a few grains per cell was present on all tissues, whereas positive control tissues had distinct, well-defined clusters of grains over infected cells. In preliminary studies, RNA/DNA hybridization assays were performed on positive control FIV-infected tissues after pretreatment with RNase to determine if proviral DNA alone could be detected. These studies showed that, in spite of various experimental conditions, only a small number of residual positive cells could be detected after RNase treatment, suggesting that the assay is not sensitive to identify cells containing a low copy number of proviral DNA alone (data not shown). These observations, along with those of other studies, determined both the high sensitivity of the RNA/DNA hybridization assay to detect productively infected cells and the limitations in its ability to identify latently infected cells.

Hematopoietic progenitor assays in methylcellulose cultures. Heparinized BM samples were washed 3 times in Iscove's modified Dulbecco's media (IMDM; Gibco, Grand Island, NY) and resuspended in IMDM with 20% heat-inactivated (56°C for 30 minutes) fetal calf serum (FCS; HyClone Laboratories, Inc, Logan, UT) before recovery of light density BM mononuclear cell (BMMNC) fractions and buffy-coat cell (BCC) fractions. The BMMNCs were isolated by centrifugation on Percoll (Sigma) at 1.070 g/mL. BM BCCs, consisting of all nucleated BM cell types, were isolated by high-speed centrifugation with subsequent hypotonic lysis of contaminating erythrocytes, as previously described.

The frequencies (number per 10^6 BMMNCs) of granulocyte/macrophage progenitors (granulocyte/macrophage colony-forming units [CFU-GM]), early erythroid progenitors (erythroid burst-forming units [BFU-E]), and late erythroid progenitors (CFU-E) were determined by colony-forming assays of BMMNCs in methylcellulose cultures, as previously described. Triplicate methylcellulose cultures contained 20% FCS, 10% heat-inactivated normal cat serum, 0.5 U/mL of recombinant human erythropoietin (Amgen, Thousand Oaks, CA), 5% heat-inactivated conditioned medium from feline embryonic fibroblasts infected with feline leukemia virus-A (FeLV-A, CCF-HeF-1), with colony numbers in cultures of BMMNCs exposed to a control solution of thymidine (4 pmoI/L; Sigma), BMMNCs in standard methylcellulose cultures of BMMNCs after a 20-minute exposure to high activity ³²P (25 Ci/mmol/L; Amersham Corp. Arlington Heights, IL), with colony numbers in cultures of BMMNCs exposed to a control solution of thymidine (4 µm/L; Sigma).

To determine whether serum from cats with acute FIV infection lacked growth-promoting activity present in normal cat serum or contained substances inhibitory to the in vitro growth of autologous or allogeneic progenitors, additional colony-forming assays were performed. For these experiments, standard methylcellulose cultures were established containing BMMNCs from infected cats with either
10% heat-inactivated autologous serum (substituted for the 10% normal cat serum) or a total of 30% FCS (without any cat serum). Additional assays were performed using BM-MNCs from normal control animals with either 10% allogeneic normal cat serum or 10% FIV cat serum.

Assays of viral expression in BM BCC cultures. BM BCCs were cultured under conditions that promoted the development of an adherent stromal cell layer, using methods previously reported. In the current studies, 3 x 10^6 BM BCCs were cultured in 25-cm² flasks (Falcon; Becton Dickinson, Lincoln Park, NJ) containing 7 mL IMDM with 12.5% FCS, 12.5% prescreened heat-inactivated horse serum (HyClone), 400 mg/L glutamine (GIBCO), 1% penicillin/streptomycin/ fungizone (GIBCO), 10^-4 mol/L Î²-mercaptoethanol, 40 mg/L myo-inositol (Sigma), 10 mg/L folic acid (Sigma), 0.26% sodium chloride, and 10^-3 mol/L hydrocortisone 21-hemisuccinate (sodium salt; Sigma). Flasks were incubated at 37°C in 5% CO_2/95% air. To determine whether in vitro FIV replication and expression were affected by antiretroviral agents or cytokines known to affect HIV replication in human monocyte/macrophages or T lymphocytes, BCC cultures were established at 3 x 10^6 or 2 x 10^6 BCCs/flask and maintained in the presence of 10 ìg/ml/azidohymidine (AZT); at a concentration known to inhibit in vitro transmission of FIV\(^2\), 2 to 5 ìg/ml recombinant human tumor necrosis factor-Î± (rhuTNF-Î±; Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) (rhuTNF-Î± is known to be physiologically active on feline BM progenitor cells\(^2\)), or 100 IU/mL rhu interleukin-2 (rhuIL-2; Hoffman-La Roche, Inc, Nutley, NJ; also known to be active on feline T lymphocytes\(^2\)). Each week, one-half volume of the culture supernatant was exchanged with fresh media. The nonadherent cells in the spent media were transferred to glass slides by cytospin centrifugation. The cytospin preparations were fixed in acetone for 10 minutes at room temperature and were stored at -20°C in a desiccator for future immunofluorescence and in situ hybridization assays. The cell-free culture supernatants were stored at -70°C for future ELISAs of FIV p24 antigen levels. Under these conditions of culture, a confluent layer of adherent cells morphologically resembling fibroblasts, macrophages, and fat cells developed by week 2 to 3. As noted in previous studies, very few, if any, hematopoietic progenitors are maintained for 2 to 3 weeks in BM BCC cultures established with 10^6 BCCs per flask.\(^2\) Nonadherent cells from these cultures consist of granulocytes, MNCs, and macrophages. In additional experiments, adherent stromal cells from 2- or 3-week-old BCC cultures were trypsinized, were passaged onto chamber slides (Lab Tek; Nunc, Naperville, IL), were allowed to adhere overnight, and then were washed and fixed in acetone (10 minutes at room temperature) before storage at -20°C in a desiccator for future immunofluorescence and in situ hybridization studies.

The relative levels of soluble FIV p24 gag antigen in the supernatants from the BM BCC cultures were determined using a commercially available antigen-capture ELISA kit (Pet Check ELISA; IDEXX, Portland, ME). Culture supernatants from Crandell feline kidney cells chronically infected with FIV Petaluma (CRFK/FIV cells) were used as a positive control. The resultant positive color changes in the ELISA wells were quantitated by measuring the absorbance values at 650 nm, using an automated microplate reader (model EL340; BioTek Instrument Inc, Winooski, VT). Serial twofold dilutions of positive control supernatants confirmed a linear relationship between p24 antigen concentration and optical densities in the ranges of 0.100 to 2.700 (data not shown). All frozen samples were thawed and assayed in one experiment to avoid the potential variation that could occur between assays performed at different times.

The expression of intracytoplasmic FIV p24 was evaluated by indirect immunofluorescence assay (IFA) of fixed nonadherent cell cytospin preparations and adherent cell chamber slide preparations from BM BCC cultures. The samples were incubated for 30 minutes at 37°C with a 1:12 dilution of the primary anti-p24 monoclonal antibody, 1C11 hybridoma supernatant (kindly provided by Fernando de Noronha, Cornell University, Ithaca, NY\(^2\)), followed by washing and incubation for 30 minutes at 37°C with a 1:30 dilution of dichlorotrizoxinylaminomethane (DTAF)-conjugated goat anti-mouse IgG Fab' fragments (Accurate Chemical and Scientific Corp, Westbury, NY). As a positive control, acetone-fixed CRFK/FIV cells were stained using the same methods. Negative control slides included uninfected BCC culture cells processed as above and infected culture cells stained with a 1:12 dilution of an irrelevant primary monoclonal antibody, an anti-FeLV gp70 antibody, C11D8 (kindly provided by Chris Grant, Pacific Northwest Research Foundation, Seattle, WA). IFA samples were evaluated on a Leitz Wetzler Ortholux II microscope with a Ploempak 2.2 vertical fluorescence illuminator (E. Leitz, New York, NY).

Adherent and nonadherent cells from 2- to 3-week-old BCC cultures were assayed for the presence of FIV nucleic acids using the in situ hybridization methods outlined above.

RESULTS

Clinical, virologic, and hematologic changes during acute FIV infection. The clinical and laboratory features of primary FIV infection in this cohort of experimentally inoculated cats resembled those previously described in detail.\(^17\) Because necropsies were performed at regular intervals, longitudinal data were not available on all animals in this study. However, a general time course of events was discernable based on changes in the surviving animals.

Palpable lymphadenopathy occurred at week 1.5 PI and persisted. Other clinical signs and symptoms, including fever, diarrhea, depression, ocular discharge, and scuffy coat, were noted at weeks 8 to 10 PI. No animal required unplanned euthanasia because of severe illness. Plasma viremia and serum antibodies against FIV gag proteins were initially detected at week 3 PI and persisted through to the study's conclusion at week 12 PI. The mean baseline absolute neutrophil count was 9.4 ± 1.1 (n = 12 cats) at week 0 of study. Neutropenia (<2.5 neutrophils/µL) was first noted in 2 of 8 surviving cats at week 6 PI; and subsequently was noted in 5 of 5 cats, 2 of 4 cats, and 2 of 2 cats at weeks 8, 10, and 12 PI, respectively. Nadir neutrophil counts of 0.2 to 1.0/µL occurred at weeks 6 to 8 PI, with a mean neutrophil count of 0.6 ± 0.1 (n = 5 cats) at week 8 PI. Hematocrits and platelet counts remained within the normal range in all cats throughout the study (data not shown). Of note, lymphocytopenia (<1500 lymphocytes/µL) occurred at weeks 8 to 10 PI.

FIV RNA expression and proviral DNA in BM cells in vivo. FIV-infected cells, detected by in situ hybridization, were first present in the BM beginning 3 weeks after inoculation, correlating with the appearance of infectious virus in the plasma and PBMCs. Infected cells were detected in BM sections from all cats that underwent necropsy at weeks 4 to 12 PI (Fig 1A and B). Viral load in the BM was low until the cats became clinically ill. Consistent with previous findings,\(^23\) the highest viral burden was found in the BM of sick cats with myeloid hyperplasia (weeks 8 and 10 PI). MNCs and megakaryocytes were both infected in all FIV-positive BM cells except in cat no. 5125 (that underwent necropsy at week 6 PI), which had infected MNCs only.

Similar numbers of positive BM cells were identified by...
RNA in situ hybridization as by RNA/DNA in situ hybridization in 6 of 9 cats with BM infection (Table 1). Thus, the majority of FIV-infected BM cells identified by these methods were productively infected. Because these assays are not sensitive enough to detect cells containing a low copy number of proviral DNA without viral RNA, the relative numbers of latently infected cells in the BM could not be determined.

Alterations in hematopoiesis during primary FIV infection. The frequencies of CFU-E, BFU-E, and CFU-GM in the BM of the animals inoculated with FIV Petaluma shifted significantly over time, above and then below the progenitor frequencies in control (uninfected) cats. At weeks 1.5 to 4 PI, the mean frequencies (number per 10^5 BMMNCs) of CFU-E, BFU-E, and CFU-GM were 508 ± 74, 143 ± 24, and 110 ± 17, respectively (n = 5 cats), compared with control values of 172 ± 24, 86 ± 26, and 44 ± 10 (P < .02.

Fig 1. In situ hybridization assays for FIV nucleic acids (RNA and proviral DNA) in BM histologic sections (A and B), in 2-week-old BM BCC culture adherent cells (C and D), and in nonadherent cell cytospin preparations (E and F). Sample preparation and methods are described in the text. No virus-containing cells were detected in BM sections (A), cultured adherent BM cells (C), or nonadherent BM cells (E) from an uninfected control cat (no. 5104). Frequent FIV-infected small MNCs and a single infected large megakaryocyte were visible among uninfected myeloid and erythroid precursors in a BM section from cat no. 5129 (that underwent necropsy at week 8 PI) (B). FIV-infected adherent monocyte/macrophage-like cells were identified among uninfected fibroblasts in BCC cultures from cat no. 5129 (D). Infected nonadherent MNCs were detected in BCC cultures from cat no. 5127 (that underwent necropsy at week 4 PI) (F). (Original magnification × 100.)
for all values; n = 3 cats; paired Student's t-test). In contrast, mean frequencies in cats studied at weeks 6 to 12 PI were 72 ± 16, 43 ± 6, and 19 ± 4, respectively (P < .01 for all values; n = 7 cats). The data obtained on individual cats studied on the day of necropsy are shown in Fig 2. Although histologic sections showed BM myeloid and lymphoid hyperplasia in some cats at weeks 8 to 12 PI, this was not consistently associated with decreased progenitor frequencies, nor were there consistent increases in the yields of total BMMNCs at weeks 6 to 12 PI (range, 25 to 70 × 10⁶; with the exception of a 244 × 10⁶ BMMNC yield from cat no. 5129 at week 8) compared with the SPF necropsy control cat (75 × 10⁶ BMMNCs). Therefore, these observations suggest that alterations in the nonprogenitor BM cell population did not result in a “dilutional” effect on the progenitor cell populations and this mechanism could not fully account for the changes in progenitor frequencies.

³HdR suicide assays on BMMNCs from cats at weeks 1.5 and 4 PI (n = 3 cats assayed) showed that the percentages of CFU-E, BFU-E, and CFU-GM in the S-phase of the cell cycle (range: 53% to 54%, 21% to 31%, and 14% to 31%, respectively) were similar to 3 age-matched uninfected controls (range: 52% to 55%, 20% to 30%, and 25% to 33%, respectively), which were similar to previously studied normal adult cats. In comparison, the percentages of progenitors in S-phase from cats at weeks 6 to 12 PI were frequently greater than the standard deviation for the mean values of current and historical control normal cats (Fig 3).

The in vitro formation of progenitor-derived colonies was not significantly affected by the presence of 10% heat-inactivated autologous serum, compared with normal cat serum, in cultures of BMMNCs from cats studied at weeks 1.5 or 3 PI (n = 3 cats). However, the mean numbers of BFU-E-, BFU-E-, or CFU-GM–derived colonies were significantly decreased (P < .05) in the presence of autologous serum from 3, 4, or 6 of 9 cats, respectively, studied at weeks 4 to 12 PI (data not shown). These data suggested either the presence of a heat-stable inhibitory activity or the absence of a normal progenitor growth-promoting activity in the sera from these animals. To evaluate these possibilities, progenitor growth in 30% FCS (without any cat serum) was compared with growth in 20% FCS and either 10% normal cat serum or 10% autologous cat serum. In the presence of 10% normal cat serum, the numbers of BFU-E–derived colonies were significantly increased (P > .05), compared with those for FCS alone, in cultures of BMMNCs from 8 of 9 cats studied at weeks 4 to 12 PI. In comparison, 10% autologous serum significantly enhanced BFU-E growth in 6 of 9 cats, with growth equivalent to FCS alone in 3 of 9 cats. Thus, the discrepancy in BFU-E–derived colony formation in normal cat serum versus autologous serum appeared to be related to a lack of erythroid growth-promoting activity in the serum of some cats with acute FIV infection. In contrast, CFU-GM–derived colony numbers were not generally increased by the presence of 10% normal cat serum, compared with those for FCS alone (only 2 of 9 cats with significantly increased numbers); whereas, for 6 of 9 cats whose BMMNC cultures were grown in the presence of 10% autologous serum, significant decreases in colony numbers (P < .05) were noted. To further define the effect of FIV cat serum on normal progenitors, additional colony assays were performed using BMMNCs from 2 different uninfected control cats with FCS alone, 10% normal cat serum (from 2 different allogeneic donors) or 10% FIV cat serum. These experiments indicated that both normal BFU-E and CFU-GM growth were significantly enhanced (P < .05) in the presence of 10% normal cat serum. Similarly, normal BFU-E growth was significantly enhanced (compared with growth in FCS alone) in 21 of 24 experiments containing 10% FIV serum. In comparison, normal CFU-GM growth was enhanced in only 8 of 24 experiments, with growth equivalent to that in FCS alone in 16 of 24 experiments. Taken together, these data suggest that serum from cats with acute FIV infection often lacks progenitor growth-promoting activities required for maximal in vitro colony formation of normal

Table 1. Summary of Clinical, Virologic, Hematologic, and BM Cell Studies of Cats Necropsied After Inoculation With FIV Pataluma

<table>
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<tr>
<th>Weeks PI (N)</th>
<th>1.5 (2)</th>
<th>3 (1)</th>
<th>4 (2)</th>
<th>6 (2)</th>
<th>8 (1)</th>
<th>10 (2)</th>
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<tr>
<td>Lymphadenopathy/systemic symptoms</td>
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<td>FIV replication in BM</td>
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<td>+</td>
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<td>BM progenitor frequencies</td>
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<tr>
<td>Percentage of BM progenitors in S-phase</td>
<td>NT</td>
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<td>Progenitor growth in autologous serum v normal cat serum</td>
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Controls (n = 3) were age-matched, uninfected animals.
Abbreviations: N, number of cats necropsied; NT, not tested; -, not detected; +, present; =, equivalent to controls; †, significantly increased (P < .05) relative to controls, ‡, significantly decreased relative to controls.

* For in situ hybridization results: +, <4 positive cells/10 × field; ++, 4 to 40 positive cells/10 × field; ++++, >40 positive cells/10 × field.
† Cat no. 5106 negative for FIV RNA.
CFU-GM and autologous BFU-E. Furthermore, serum from cats at weeks 4 to 12 PI frequently contains an inhibitory activity that suppresses the growth of autologous, but not normal, progenitors of the myeloid lineage.

In vitro expression of FIV in cultures of BM BCCs. Soluble FIV p24 antigen was detectable in the supernatants of 1- to 3-week-old cultures of BM BCCs isolated from cats at weeks 4 to 12 PI (Fig 4A). These cultures were established with essentially all nucleated BM cell types and were grown under conditions that favored the growth of a heterogenous adherent layer of fibroblasts, fat cells, macrophages, and reticular cells. No detectable p24 antigen was found in the supernatants of cultures of BCCs from cats at weeks 1.5 or 3 PI (n = 3 cats). Maximal in vitro p24 production occurred in cultures of cells from cats at week 10 PI (Fig 4A). There were no morphologic differences (ie, evidence of syncytial formation or obvious cytopathic effects) in the adherent cells from FIV antigen-producing cultures as compared with those from antigen-negative cultures. Immunoassays for intracytoplasmic FIV p24 failed to identify productively infected adherent cells from 2- or 3-week-old BCC cultures from cats at weeks 4 to 8 PI. However, in situ hybridization assays did identify infected adherent cells (Fig 1D). These round, infected cells morphologically appeared to be macrophages; whereas the fibroblasts were not infected. Both in situ hybridization and IFA of nonadherent cell cytoplasm preparations from 1- to 3-week-old cultures of BCCs from cats at weeks 4 to 12 PI identified cells with FIV nucleic acids (Fig 1F) and intracytoplasmic p24 (data not shown), respectively. Antigen-positive cells were more frequent in 1-week-old cultures. These cells were small to medium size with round or reniform nuclei and occasionally with cytoplasmic vacuoles. Morphologically, these cells resembled lymphocytes, monocytes, or small macrophages. The majority of cells in the nonadherent cell fractions of the 1-week-old cultures were granulocytes, whereas the 3-week-old cultures predominantly contained large, vacuolated macrophages.

The presence of AZT resulted in only a 25% or less reduction in maximal soluble p24 production in BCC cultures (see example in Fig 4B), suggesting that in vitro transmission of FIV was not a major factor contributing to the viral replicative activity. Addition of rhTNF-α to the cultures had either no effect (at 3 × 10^6 BCCs/flask) or was associated with a decrease in p24 production (in cultures seeded with 2 × 10^6 BCCs/flask; see Fig 4B). In these cultures, rhTNF-α induced early macrophage differentiation among the nonadherent cell populations as compared with those for control flasks. In contrast, addition of rhIL-2 resulted in a 1.5-fold to fourfold increase in soluble p24 levels in cultures seeded with 2 × 10^6 BCCs (Fig 4B). The presence of rhIL-2 did not alter the relative populations of nonadherent neutrophils and MNCs. Together, these observations suggest that FIV-infected cells are present in the BM BCC population and that these cells actively replicate FIV in vitro for at least 2 to 3 weeks. Although the phenotype of the infected cell population was not defined, high levels of TNF-α or IL-2 modulated the viral replicative activity among the heterogenous population of BM cells in these cultures. This effect was potentially due to either direct effects of the cytokines on the expression of the virus or indirect effects related to alterations in the proliferation and/or differentiation status of susceptible host cells in culture.

DISCUSSION

This study shows that the severe neutropenia induced by experimental acute infection with FIV Petaluma is associated
with significant alterations in the frequencies of BM hematopoietic progenitor cells, increases in progenitor cell cycle status, and suboptimal in vitro progenitor growth in the presence of autologous serum. Furthermore, these changes develop shortly after FIV-infected cells are initially detected in the BM. The sequence of virologic, humoral, hematologic, and hematopoietic events that occur after inoculation with FIV Petaluma are summarized in Table 1.

Generalized lymphadenopathy was present within 2 weeks PI and persisted. Clinical signs of illness, including fever, diarrhea, depression, ocular discharge, and scruffy coat, were prominent at weeks 8 to 10 PI, when neutrophil counts were at their nadir. Plasma viremia and antiviral antibodies developed by week 3 PI, preceding the onset of neutropenia by 3 to 5 weeks. Absolute lymphopenia frequently accompanied neutropenia at weeks 8 to 10 PI, analogous to the transient lymphopenia noted shortly after the onset of systemic clinical symptoms in patients with primary HIV infection. Notably, anemia and thrombocytopenia did not develop in these animals, which is consistent with findings from previous studies of experimental acute FIV infection. This pattern of isolated neutropenia is in contrast to the transient pancytopenia that accompanies the symptomatic acute phase of horizontally transmitted infection with FelV, an oncornavirus with a broad host cell range that includes hematopoietic progenitors. Because previous studies of cats infected with FIV and of patients infected with HIV suggest that hematopoietic progenitors/precursors are not major targets of lentiviral infection in vivo, mechanisms involved in BM suppression are more likely related to the effects of exogenous virus or viral antigens on progenitors and/or to viral effects on BM accessory cells (T lymphocytes, macrophages, and stromal cells).

The frequencies of CFU-E, BFU-E, and CFU-GM were significantly decreased in the majority of cats studied at weeks 6 to 12 PI (Fig 2). Impaired CFU-GM maturation could have resulted in the observed neutropenia. The lack of anemia in these cats likely was because of the long normal life span of erythrocytes in the circulation and the absence of mechanisms that shorten red blood cell survival (ie, hemorrhage or hemolysis). Because histologic sections of some cats studied at weeks 8 to 12 PI showed BM myeloid and lymphoid hyperplasia, decreases in progenitor cell frequencies secondary to the dilutional effect of nonprogenitor BM...
cells in culture could not be ruled out in all cases. However, this association was not uniform and was not accompanied by consistent increases in the yields of BMMNCs. The temporal association of decreased progenitor frequencies occurring 2 weeks after the appearance of FIV-infected BM cells (Table 1) more strongly suggests that progenitor viability and/or proliferation were impaired by viral-mediated factors within the BM microenvironment.

Lentivirus infections in other species have been associated with alterations in hematopoietic progenitor frequencies. For example, decreases in the frequencies of BM CFU-GM and BFU-E occur during acute and chronic stages of infection with the simian immunodeficiency virus of macaques (SIVmac), and these changes are associated with the ability to recover virally infected BM-derived macrophages in culture. In addition, decreased in vitro colony formation by PB or BM BFU-E, CFU-GM, or multipotential granulocyte, erythroid, megakaryocyte, and macrophage progenitors (CFU-GEMM) has been observed in many, but not all, studies of HIV-seropositive patients. In some cases, impaired progenitor growth in culture was independent of the clinical stage of HIV infection or the presence of PB cytopenias. Removal of accessory T lymphocytes from the PB or BMMNCs before culture has been reported to improve the abnormal in vitro colony formation, suggesting that progenitor growth is suppressed by HIV-infected or dys-

Fig 4. Production of soluble FIV p24 antigen in cultures of BM BCCs from cats experimentally infected with FIV Petaluma. Relative levels of p24 were determined by ELISA absorbance values (at 650 nm) in supernatants of cultures containing $3 \times 10^6$ cells under standard conditions (A) or containing $2 \times 10^6$ cells with added agents (B). Data points in (A) represent mean values from 1- to 3-week-old cultures of cells from 2 cats at weeks 1.5, 4, 6, 10, and 12 PI with FIV and from 1 cat each at weeks 3 and 8 PI. Data points in (B) represent mean levels in parallel cultures of BCCs from cat no. 5127 (that underwent necropsy at week 4 PI) maintained in standard control conditions or in standard conditions plus either 10 $\mu$mol/L AZT, 100 U/mL rhuIL-2, or 4 ng/mL rhuTNF-α. Assays of two-fold serial dilutions of positive control culture supernatants showed a linear relationship between p24 antigen concentration and optical density (OD) in the ranges of 0.100 to 2.700 (data not shown).
regulated T cells. In one study, the addition of antisense oligonucleotides directed against HIV regulatory gene sequences increased colony formation in cultures of adherent cell-depleted BMMNCs but not in cultures of CD34+-enriched BM cells, indicating that suppression of viral expression in vitro (presumably in an infected BM accessory cell) enhanced progenitor growth. These observations, together with our data, suggest that lentivirus-infected BM accessory cells and/or their extracellular products could impair progenitor survival or differentiation.

The percentages of committed erythroid and myeloid progenitors in the DNA synthetic phase of the cell cycle were frequently increased in cats at weeks 6 to 12 post-FIV infection (Fig 3). In comparison, BM progenitors from cats with earlier primary FIV infection (Fig 3) or asymptomatic, chronic FIV infection have unimpeded cell cycle kinetics associated with increased (Fig 2) or normal progenitor frequencies, respectively. Thus, the increased progenitor cell growth fractions detected during the neutropenic stage of primary infection could be related to compensatory mechanisms responding to the decreased circulating and/or progenitor cell pools. Alternatively, enhanced progenitor proliferation could be secondary to accessory/stromal cell cytokine production induced directly by exposure to viral protein or induced by inflammatory mediators (eg, IL-1 or TNF-α) released by infected or viral antigen-exposed monocyte/macrophages. The observed increase in cell cycle kinetics of myeloid progenitors could explain, in part, the myeloid hyperplasia noted in the BM of some cats at weeks 8 to 10 PI. Because BM hyperplasia and dysplasia are found in cats with symptomatic, chronic FIV infection and in patients with chronic HIV infection, similar mechanisms may occur in late stages of both of these lentivirus infections.

In vitro growth-inhibitory activity against autologous but not normal CFU-GM was detected in the heat-inactivated serum of 6 of 9 cats studied at weeks 4 to 12 PI. This activity was not further characterized but was associated with a loss of the progenitor growth-promoting activity that is normally present in cat serum. Because heat-inactivated serum from cats with asymptomatic chronic FIV infection does not inhibit the in vitro growth of autologous or allogeneic BM progenitors, the inhibitory serum activity detected during acute infection is not likely to be caused by antiviral or other antibodies. Furthermore, although antineutrophil antibodies have been reported in HIV-seropositive patients and in other patients with diverse, acute viral infections, the presence of such antibodies has not been closely correlated with PB neutropenia in these cases. Thus, the serum activity against autologous feline CFU-GM is more likely to be related to the virus, viral antigens, and/or humoral inhibitory substances that may act on a subset of CFU-GM that are rendered particularly susceptible to growth inhibition during acute viremia.

FIV nucleic acids were detected in BM megakaryocytes and MNCs by in situ hybridization assays of histologic sections of cats at weeks 4 through 12 PI (Fig 1 and Table 1). Similar numbers of positive cells were detected by RNA/DNA hybridization in 6 of 9 cats, indicating productive infection in many cells within the BM. The presence of infected cells in vivo correlated with the detection of soluble FIV p24 in cultures of BM BCCs from these same animals (Fig 4). Intracytoplasmic p24 and FIV nucleic acids were detected by indirect IFA (data not shown) and in situ hybridization studies (Fig 1), respectively, in nonadherent cells from viral-producing cultures. The infected MNCs morphologically resembled small monocyte/macrophages and lymphocytes. Large, reactive nonadherent macrophages were uniformly negative for intracytoplasmic p24, as were adherent macrophages and fibroblasts from these cultures. However, adherent monocyte/macrophage cells were positive for FIV nucleic acids as determined by in situ hybridization (Fig 1). These observations suggest that FIV-infected monocyte/macrophage cells are frequently found in the BM of cats with primary FIV infection and that these infected cells can be propagated in vitro. Furthermore, active viral expression, or latent infection, may be related to the differentiation and/or activation status of host BM monocytes, consistent with observations of virus expression in FIV-infected peritoneal macrophages.

Addition of rhuIL-2 to the BM BCC cultures resulted in a maximal fourfold increase in soluble p24 levels (Fig 4B), suggesting that active viral replication occurred, at least in part, in infected BM T lymphocytes in these cultures. It was not further determined whether the IL-2 effect was caused by expansion of the T-lymphocyte population or by enhanced viral replication and/or transmission. In contrast, FIV p24 production was either unchanged or decreased by the addition of rhuTNF-α to the BCC cultures (Fig 4B). Exposure of FIV-infected feline fibroblastic cells, but not of uninfected cells, to TNF-α enhances apoptotic cell death and inhibits viral replication in culture. Therefore, a similar mechanism may explain our observations in BCC cultures. This response differs from that of HIV-infected cells in that TNF-α upregulates viral expression in both infected T-lymphocyte and macrophage human cell lines. This effect is mediated by the activation of the cellular transcription factor NF-B, which subsequently binds to specific sequences in the enhancer region of the HIV proviral long terminal repeat (LTR). A putative NF-κB binding site has been identified in the LTR of one FIV molecular clone but not in that of another clone, suggesting that the effects of TNF-α on FIV replication could be virus strain-specific and complex.

In conclusion, these studies show that the severe neutropenia induced by experimental acute FIV infection is associated with the appearance of virus-infected BM accessory cells and alterations in hematopoietic progenitor frequencies, cell cycle kinetics, and in vitro growth in autologous serum. Collectively, these data suggest that progenitor survival or growth could be impaired within the BM microenvironment by the virus, viral antigen, and/or BM accessory cells dysregulated by viral infection or antigens. The similarities between these observations and abnormalities in hematopoiesis associated with chronic HIV and SIV infection support the hypothesis that studies of host-viral interactions during experimental acute infection with FIV could provide insights into the pathogenesis of lentivirus-associated BM suppression during late stages of infection.

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Marrow accessory cell infection and alterations in hematopoiesis accompany severe neutropenia during experimental acute infection with feline immunodeficiency virus

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